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Dates covered:
July 1, 1972 - December 31, 1972

The Influence of Extended Exposure to Cigarette Smoke on Pulmonary Resistance to Infection as Related to Alveolar Macrophage and Mucociliary Function.

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Contents of Progress Report No. 7

On May 28, 1971, I received notification from W. T. Hoyt, Executive Vice President of the Council for Tobacco Research-USA, Inc., that the application for our research grant entitled "The Influence of Extended Exposure to Cigarette Smoke on Pulmonary Resistance to Infection as Related to Alveolar Macrophage and Mucociliary Function" was approved for support. The grant was funded starting July 1, 1971. In the meantime, I accepted a new position at the present institution and the first year of support was terminated on January 31, 1972. The move took place in February and the first year of support was continued in March 1972. A renewal application was submitted on March 28, 1972, and the second year of support began on July 1, 1972, at Long Island Jewish-Hillside Medical Center/Queens Hospital Center Affiliation. The present request is, as indicated, for renewal beginning July 1, 1973. Although progress reports have been submitted for each year of past support, the most complete ones are Progress Reports Numbers 4 and 5, covering the periods 1969 to 1970 and 1970 to 1971. The last detailed progress report (No. 6) was submitted on March 28, 1972, covering the grant period of 1971 to 1972. In keeping with the recent changes in the renewal dates for CTR Grants and the recent relocation of my research laboratories, the present progress report (No. 7) will include a summary statement of previous detailed reports and this will be related to a status report of my current research efforts from July 1972 to the present.

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Summary Statement* of Progress Reports Nos. 5 and 6

There is ample clinical and experimental evidence which demonstrates that the normal lung tends to maintain a sterile atmosphere; namely, it is free of viable bacteria. Evidence for the antibacterial activity of the broncho pulmonary tissue of human beings and experimental animals, respectively, resides in the observations that bronchial secretions of normal human subjects are sterile and that bacteria deposited in the lungs of animals are rendered non-viable within hours.

Although several factors undoubtedly contribute to the clearance of foreign material deposited in the lungs, the mucociliary apparatus and alveolar macrophage defense systems comprise the major defenses of the respiratory tract. Attention has been focused on the alveolar macrophage system by studies completed in this laboratory, as well as others, which demonstrate that alveolar macrophages are mobilized in response to the inhalation of viable and non-viable bacteria, cigarette smoke and carbon dust. Mobilization provides a mechanism by which large numbers of macrophages are made available to sites of pulmonary insult and therefore may be intimately associated with the processes that permit the lung to maintain a sterile atmosphere.

*The materials, methods and data have been presented in detail in Progress Reports numbers 5 and 6.

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The studies reported in Progress Reports Numbers 5 and 6 are concerned with the influence of acute exposure to cigarette smoke on pulmonary resistance to bacterial infection and utilizes methods for investigating the quantitative aspects of pulmonary defense as related to the alveolar macrophage. Specifically, the influence of smoke inhalation was assessed in terms of its effect on the numbers of available macrophages, cell viability and antibacterial activity. In addition, by utilizing an in vitro phagocytosis system and in vivo and in vitro conditions of smoke exposure, it has been possible to evaluate the following aspects of the antibacterial activity of rabbit alveolar macrophages in the normal situation and during exposure to cigarette smoke: (1) the influence of culture conditions and serum factors on antibacterial activity in the normal situation and during exposure to cigarette smoke, (2) the importance of changes in culture media pH induced by cigarette smoke on macrophage function, (3) the role of increased macrophage numbers (mobilization) as a determinant of the in vitro antibacterial capacity of an alveolar macrophage population in the presence and absence of cigarette smoke, (4) the influence of alveolar macrophage activity on the toxicity of a smoke solution (5) the antiphagocytic effect of in vivo smoke exposure on the acellular fraction (pulmonary lavage solution) of lung harvests, (6) the relative effect of whole smoke and the gas phase of whole smoke on the antibacterial properties of macrophages (7) the reversibility of changes in macrophage function induced by exposure to whole cigarette smoke under in vitro and in vivo conditions of smoke exposure and (8) to compare the effects of whole cigarette smoke on the antibacterial activity of macrophages under in vitro and in vivo conditions of smoke exposure.

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Materials and Methods

A. In Vitro Phagocytosis System: Alveolar macrophages harvested from rabbits were placed in tissue culture flasks containing Hanks' solution supplemented with normal rabbit serum and challenged with S. aureus. At specific intervals after bacterial challenge, the reduction in the number of viable staphylococci present in the extracellular fraction of tissue culture preparations was used to determine the percent bacteria phagocytized while the number of viable bacteria cultured from monolayers (macrophage fraction) provided estimates of the percent intracellular survival of ingested bacteria. Direct studies of the bactericidal capacity of macrophage cultures were also performed in which the ingestion and post-ingestion phases of staphylococcal inactivation were dissociated by washing monolayers after bacterial challenge. A standard pour plate technique was used to obtain bacterial counts from the extracellular and macrophage fractions immediately after bacterial challenge and at various intervals, thereafter. Under these conditions, it is possible to quantitate the following parameters of alveolar macrophage function: (a) the percent bacteria phagocytized by macrophages, (b) the percent phagocytized bacteria surviving within macrophages, (c) the percent bacteria killed by macrophage cultures over a 1.5 hour test period and (d) a direct assessment of bactericidal capacity of macrophages. The methods, procedures and details of the in vitro phagocytosis system have been presented in detail in Progress Report No. 1 for Grant #547-B.

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B. Conditions of In Vitro Smoke-Exposure: A commercial brand of non-filtered cigarette was attached to a 30 ml. syringe by a rubber tube and smoke was produced by withdrawing the barrel of the syringe at a rate of approximately 18 ml. per second. Six successive "puffs" of cigarette smoke were introduced into the syringe and emptied by removing a rubber tube containing the lighted cigarette. The smoke from the seventh puff was introduced into the tissue culture flask with a sterile hypodermic needle. The protocol of each study included control flasks containing only bacteria and alveolar macrophages challenged with bacteria respectively. Under these conditions, both categories of tissue culture preparations were exposed for 1.5 hours to whole cigarette smoke and whole smoke drawn through a glass fiber filter.**

C. Conditions of In Vivo Smoke-Exposure: A smoke generating apparatus*** was used to deliver puffed cigarette smoke under controlled conditions. In the studies reported herein, the apparatus was adjusted to deliver a 35 ml. puff of 2 seconds duration from a commercial brand of non-filtered cigarettes. The time interval between puffs from a single cigarette was set at 58 seconds. The instrument is equipped with a rotating disc which can accommodate 30 cigarettes at one time, so that it is possible to maintain a continuous stream of puffed smoke generated at a rate of 1 L/min. The initial smoke delivered by the cigarettes is diluted and transported across an animal exposure chamber by a secondary air flow. Control animals are placed in a plexiglass chamber and exposed to a secondary air flow. In the present experiments, smoke-exposed and control animals were exposed to whole cigarette smoke and a secondary air flow, respectively, for 1.5 hours.

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Discussion of Results and Significance of the Data

In the reported studies, smoke inhalation proved an effective means of inducing a macrophage response. Specifically, smoke inhalation provoked an increase in the numbers of alveolar macrophages available to the lung without any adverse effect on cell viability. In addition, it did not stimulate the migration or proliferation (mobilization) of other phagocytic cells such as polymorphonuclear leukocytes. These observations demonstrate that one of the immediate effects of smoke inhalation on the cellular defense mechanisms of the respiratory tract is a direct stimulation of the alveolar macrophage defense system. The data further suggests that the presence of increased macrophages may play a central role in protecting the lung against smoke toxicity. To postulate such a protective role for these cells is supported by studies performed under in vitro conditions of smoke exposure and presented in Progress Report No. 6 which demonstrate the following: (1) increased numbers of macrophages reduce or limit the adverse effect of cigarette smoke on the antibacterial activity of a macrophage population in vitro and (2) alveolar macrophages are capable of inactivating toxic components present in a smoke solution that impair the antibacterial activity of normal alveolar

**Cambridge glass fiber filter - Phipps and Bud, Richmond, Va.
***The details and calibration data of the smoke-exposure system has been presented in detail in Progress Report No. 6.

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macrophages in vitro.

The data obtained from a direct investigation of the phagocytic and bactericidal properties of alveolar macrophages harvested from smoke-exposed animals (in vivo conditions of smoke-exposure) has provided additional pertinent information. As demonstrated by the pulmonary lavage studies, exposure to cigarette smoke caused an accumulation of "toxic substances" that can be washed out of the lungs and inhibit the antibacterial activity of alveolar macrophages in vitro. However, smoke inhalation did not permanently impair the antibacterial activity of the alveolar defense system. Specifically, smoke-exposure caused an impairment of the phagocytic and bactericidal powers of macrophage harvests that was readily reversible within hours. The fact that macrophage function was restored under in vitro conditions of recovery; namely, by multiple washing and incubation of macrophage in a smoke-free environment demonstrates the capacity of the alveolar macrophage system to cope with cigarette smoke and the importance of in vivo processes which effectively remove inhaled products of cigarette smoke from the respiratory tract. To correlate with this, the adverse effect of smoke inhalation was reversed under in vivo conditions of recovery, namely, by housing smoke-exposed animals in a smoke-free room prior to obtaining lung harvests. Collectively, these observations suggest that the respiratory tract responds to the presence of cigarette smoke, as well as other foreign materials in a similar manner; namely, pulmonary defense mechanisms are activated and respond by removing and/or inactivating smoke products deposited in the lung. It appears reasonable to assume that the efficacy of this response is facilitated by the in vivo mobilization of increased macrophage numbers in relation to the inhalation of cigarette smoke. Such an interpretation of the data would be in keeping with experimental evidence previously cited in this report which demonstrates that alveolar macrophages may play a key role in protecting the lung against the toxic effects of cigarette smoke.

Studies performed under in vitro conditions of smoke-exposure have served to provide: (1) the laboratory techniques necessary for more meaningful studies of the effect of cigarette smoke on the living and functioning lung and (2) a better understanding of the conditions of bacterial challenge and smoke-exposure that influence the antibacterial properties of alveolar macrophages. As demonstrated by studies presented in Progress Reports Numbers 5 and 6, the antibacterial capacity of a macrophage population in the absence and presence of cigarette smoke is enhanced by the presence of increased numbers of alveolar macrophage. This observation and the detoxification data presented earlier, support published reports which have focussed attention on the importance of alveolar macrophage mobilization as a pulmonary defense mechanism against inhaled infectious and toxic agents. Results of other studies performed under in vitro conditions of smoke-exposure indicate that the local conditions necessary for effective antibacterial activity by normal and smoke-exposed macrophages differ. In this regard, the antibacterial activity of macrophages during smoke-exposure was significantly influenced by the concentration of serum in the culture medium. This effect could not be accounted for by the presence or absence of an added carbon source (glucose) in the culture medium or the increased acidity of the culture medium induced by cigarette smoke. The fact that exposure to cigarette smoke interfered with the antibacterial activity of alveolar macrophages only at serum concentrations of 2.5% or less indicates that serum factors may pro-

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tect against smoke toxicity. This may be related to specific immunological and/or nutrient components of serum. In addition, it has also been observed that the protective action of serum is reduced if cigarette smoke is introduced prior to bacterial challenge. Specifically, prior exposure of macrophage cultures to cigarette smoke reduced the amount of cigarette smoke necessary to suppress antibacterial activity. This difference may be a reflection of the long term effects of cigarette smoke on a static macrophage population and probably relates to several factors including the effect of extended exposure to cigarette smoke on: (1) serum factors which promote and sustain effective macrophage function and (2) the physiological state of macrophages at the time of bacterial challenge. Macrophages obviously respond to the presence of cigarette smoke by removing and/or detoxifying smoke products in their environment. The fact that the adverse effects of in vitro smoke-exposure was partially reversed by multiple washing of macrophage cultures demonstrates the beneficial results derived from reducing the amount of smoke in contact with the alveolar cells and the importance of the cleansing action of increased numbers of macrophages (mobilization) and mucociliary activity in the live and functioning lung. Data has also been presented which clearly demonstrates that exposure to whole smoke drawn through a glass fiber filter reduced the adverse effect of in vitro smoke-exposure on the antibacterial activity of macrophages. Specifically, filtered whole smoke did not interfere with phagocytic activity but continued to impair bacterial killing. These observations suggest that the particulate and gas phases of whole smoke may have separate effects on macrophage function.

Marked differences in the effect of in vivo and in vitro smoke-exposure on macrophage function have also been noted. In vitro conditions of smoke-exposure produced an impairment of antibacterial activity that was not totally reversed by multiple washing and incubation of macrophages in smoke-free tissue culture flasks. The differences in the effects of in vivo and in vitro smoke-exposure on macrophage function may be attributable to several factors including: (1) differences in the amount of smoke that macrophages were exposed to under both experimental conditions and (2) the static and unphysiological conditions that prevail under in vitro conditions of smoke-exposure. The latter interpretation of the data would be in keeping with the mobilization and detoxification data reported herein. Since it is apparent that in vitro findings may not parallel the in vivo process, studies will be performed to determine the influence of acute and extended exposure to whole and filtered smoke on pulmonary defense as related to alveolar macrophage function under in vivo conditions of smoke-exposure.

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Status Report of Research Covering Grant Period 7/1/72 to 12/31/72

In keeping with the experimental design outlined in the original grant request studies are in progress to evaluate the influence of extended exposure to cigarette smoke of pulmonary resistance against inhaled bacteria. The latter studies involve housing mice in our laboratory for long periods of time during exposure to cigarette smoke and prior to bacterial aerosol studies. For these reasons it was necessary to determine the influence of the laboratory environment on the sterility of the broncho-pulmonary tree of normal mice and to standardize all procedures necessary to perform meaningful bacterial aerosol and clearance studies. In addition, if animal age is a significant factor, it would be essential to control age in any experiment designed to assess the effect of cigarette smoke on pulmonary defense against inhaled bacteria.

Materials and Methods****

Animals: White male Swiss Webster mice (Charles River - CR-1 strain) were used in all experiments. The animals were housed in plastic cages, 4 per cage, and were permitted access to food and water ad libitum. The lungs of this strain of mice are normally free of detectable viable bacteria. To avoid exogenous bacterial contamination, the animals are shipped to the laboratory in filtered crates.

Aerosol Challenge: A coagulase positive strain of Staphylococcus aureus (FDA 209, phage type 42D) is prepared for aerosolization in the following manner. The second of two successive 12 hour trypticase soy broth cultures are harvested by centrifugation at 6,000 rpm for 20 minutes. The supernatant fraction is discarded and the remaining bacterial pellet is washed with a phosphate buffer (11.36g Na_2HPO_4 and 2.72g KH_2PO_4 per liter) at pH 7.4. Desired concentrations are achieved by resuspending the pellet in appropriate volumes of the phosphate buffer. The number of viable staphylococci present in bacterial suspensions is determined from nutrient agar pour plates incubated at 37° C for 48 hours.

The bacterial aerosol exposure apparatus includes: (a) an aerosol-generating system, (b) a mixing chamber, (c) an animal exposure chamber and (d) a filter-discard system. Aerosols of staphylococci are generated from a buffered suspension of bacteria contained in eight DeVilbiss #40 glass nebulizers. The initial spray from each nebulizer is directed into a mixing chamber with an interposed baffle to remove large droplets and mixed with a secondary airflow of 100 cubic feet per minute which serves to dilute, dry

****The methods of aerosol formation and bacterial aerosol exposure system have been previously described. G.A. Laurenzi and J.J. Guarneri, 1966, Amer. Rev. Respiratory Diseases Vol. 93: 134-141.

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and direct the bacterial aerosol through a large animal exposure chamber. The volume of test bacterial solution nebulized is recorded and the concentration of viable airborne bacteria in the animal exposure chamber is determined from pour plates made of air samples collected with glass impinger samplers containing 20 ml of the phosphate buffer. Particle size determinations are made with an Anderson sampler modified by the manufacturer to separate the aerosol into 8 particle size ranges. A sample of exposure chamber air is drawn through the sampler and the number of viable staphylococci collected in each stage is determined by direct colony count or with the aid of a microscope after a short period of incubation at 37° C.

Bacterial Clearance Studies: Studies of lung clearance are performed in which mice are exposed for 30 minutes to aerosols of phosphate-buffer suspensions of viable staphylococci. Immediately after exposure (0 time) one group of mice is killed and the remainder are sacrificed at various intervals post-aerosol exposure. The lungs are removed aseptically; individually ground in glass homogenizers containing nutrient broth; and bacterial counts obtained from nutrient agar pour plates incubated for 48 hours at 37°C. Under these circumstances, it is possible to determine the number of viable bacteria present in the lungs at 0 time (bacterial deposition) and at each designated interval during the post-aerosol exposure period. From these data lung clearance rates are calculated by expressing the number of bacteria remaining in the lung at each interval after aerosol exposure as a percent of the number deposited at 0 time and subtracting this value from 100%. The term clearance as used in these studies refers to the numbers of bacteria whose presence in a culturable state can no longer be demonstrated.

Results

A. The Influence of the Laboratory Environment on the Sterility of the Respiratory Tract of Test Mice: For this purpose mice were housed in the laboratory for 4 weeks and different groups of mice sacrificed immediately upon delivery to the laboratory and at weekly intervals thereafter. The presence or absence of bacteria was determined from pour plates made of lung homogenates.

As previously stated, the lungs of this strain of Charles River Mice are normally free of viable bacteria. The data presented in Table I demonstrates that conditions of housing and laboratory environment did not cause pulmonary colonization of bacteria. This is indeed remarkable since ambient conditions were provided and therefore the inhalation of airborne bacteria present in the environment and the aspiration of bacteria normally present in the oral-pharyngeal regions is an inevitable occurrence. The data also emphasizes the remarkable capacity of the lower respiratory tract to maintain a sterile atmosphere and the efficacy of the antibacterial mechanisms available to normal lungs.

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Conclusions: From the data obtained to date it is also reasonable to assume that the conditions of animal quarters established at Queens Hospital Center are adequate and provide appropriate controls for studies of the influence of extended exposure to cigarette smoke on the normal defense mechanisms of the pulmonary system.

B. Aerosol Studies - The Particle Size Distribution of the Bacterial Aerosols Used to Deposit Staphylococci in the Lungs of Test Mice: Particle size is a major determinant of the probable site of deposition of inhaled airborne particles in the respiratory tract. The importance of particle size resides in the fact that particles which are deposited on the bronchial tree are subjected to the cleansing action of the mucociliary apparatus and those which deposit in alveoli are phagocytized by alveolar macrophages. Therefore, the mode of clearance is dependent upon the site of deposition, which is in turn a function of particle size. It is also apparent that particle data is mandatory for standardization of test conditions from one experiment to the next and the interpretation of data from multiple studies performed over a long period of time.

Particle size determinations were made 15 minutes after aerosolization of phosphate-buffered suspensions of staphylococci. The cummulative percent of viable bacteria collected on each impaction stage of the Andersen sampler was plotted against particle diameter on logarithmic probability paper using the 50% cut-off of that stage. A line of best fit was drawn by eye and the count median diameter (CMD) of the aerosol was derived by reading the size corresponding to the 50% intercept on the graph. As shown in Figure 1, the data obtained from a single experiment indicates that the bacterial aerosol had a CMD of 1.9 μ and a geometric standard deviation of 1.6 μ. In five separate aerosol studies the average CMD, and cumulative percent particles $\leq$ 3.3 μ, was 1.9 μ and 86%, respectively, through exposure chamber concentrations of 3.6×10^3 to 1.1×10^7 staphylococci per ft^3 of air (Table II).

Conclusions: The data demonstrates that the aerosol system produces bacterial aerosols of controlled concentrations and particle size from which predictable numbers of staphylococci can be implanted in the lungs of mice.

C. Bacterial Clearance Studies: Lung clearance studies were performed to provide the following information: (1) the influence of the laboratory environment on the deposition and clearance of large numbers of staphylococci from the lungs of mice and (2) the relationships which may exist between animal age, body weight and the number of bacteria deposited by aerosol exposure, and clearance rates. This information is necessary to plan and interpret the results of studies in which animals will be exposed to cigarette smoke over a 4 or 5 week period prior to bacterial aerosol challenge.

In some studies groups of mice were challenged with staphylococcal aerosols for 30 minutes 1 day after arriving at the laboratory, and in other experiments mice were exposed to bacterial aerosols 1, 2, 3 and 4 weeks after receipt. Bacterial deposition and 4 hour clearance rates were determined as outlined under Materials and Methods. In separate studies, animal weights recorded prior to bacterial aerosol exposure were correlated

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with mean deposition values. The results of a study in which animal weights and mean deposition values were recorded are presented in Table III. As shown in Table III, exposure of mice ranging in weight from 24.2 g to 50.0 g to the same concentration of airborne Staphylococci resulted in a weight related increase in the number of bacteria deposited in the lungs. Bacterial deposition values of 70,000, 120,000 and 168,000 were achieved in mice weighing 24.2g, 37.2g and 50.0g, respectively.

The data covering the influence of the laboratory environment and animal age on bacterial clearance is presented in Table IV. Bacterial clearance by animals housed in the laboratory from 1 day to 4 weeks was essentially the same.

Conclusions: The data demonstrates the importance of monitoring body weight and environmental conditions during long term studies and suggests that the laboratory facilities at Queens Hospital are adequate for the experiments proposed in the original grant request.

Comments

The methods described herein offer a predictable quantitative technique for studying pulmonary resistance to airborne infection. The method includes: (1) the production of bacterial aerosols of controlled concentration and particle size, (2) the deposition of predictable numbers of organisms in the lungs of test animals (3) the demonstration of rapid clearance of bacteria in the normal situation and (4) the measurement (assay) of significant changes in clearance produced by experimental conditions. The high degree of predictability offered by the aerosol system makes it especially adaptable as a reference challenge-response system to study the influence of extended exposure to cigarette smoke on pulmonary resistance to infection. In this regard, the feasibility of the proposed long term studies has been established by the data obtained to date which indicates that the laboratory environment and animal age do not compromise the pulmonary defense mechanisms of the test animals in our experimental model.

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Table 1. The Effect of Exposure to Laboratory Conditions on the Sterility of the Murine Lung.

No. of Animals	No. of Days Housed in Laboratory	No. of Animals from which Bacteria* were isolated from their lungs.
30	1	1
30	7	1
30	14	0
30	21	1
30	28	2

*The number of organisms isolated ranged from 1 to 10 organisms/lung.

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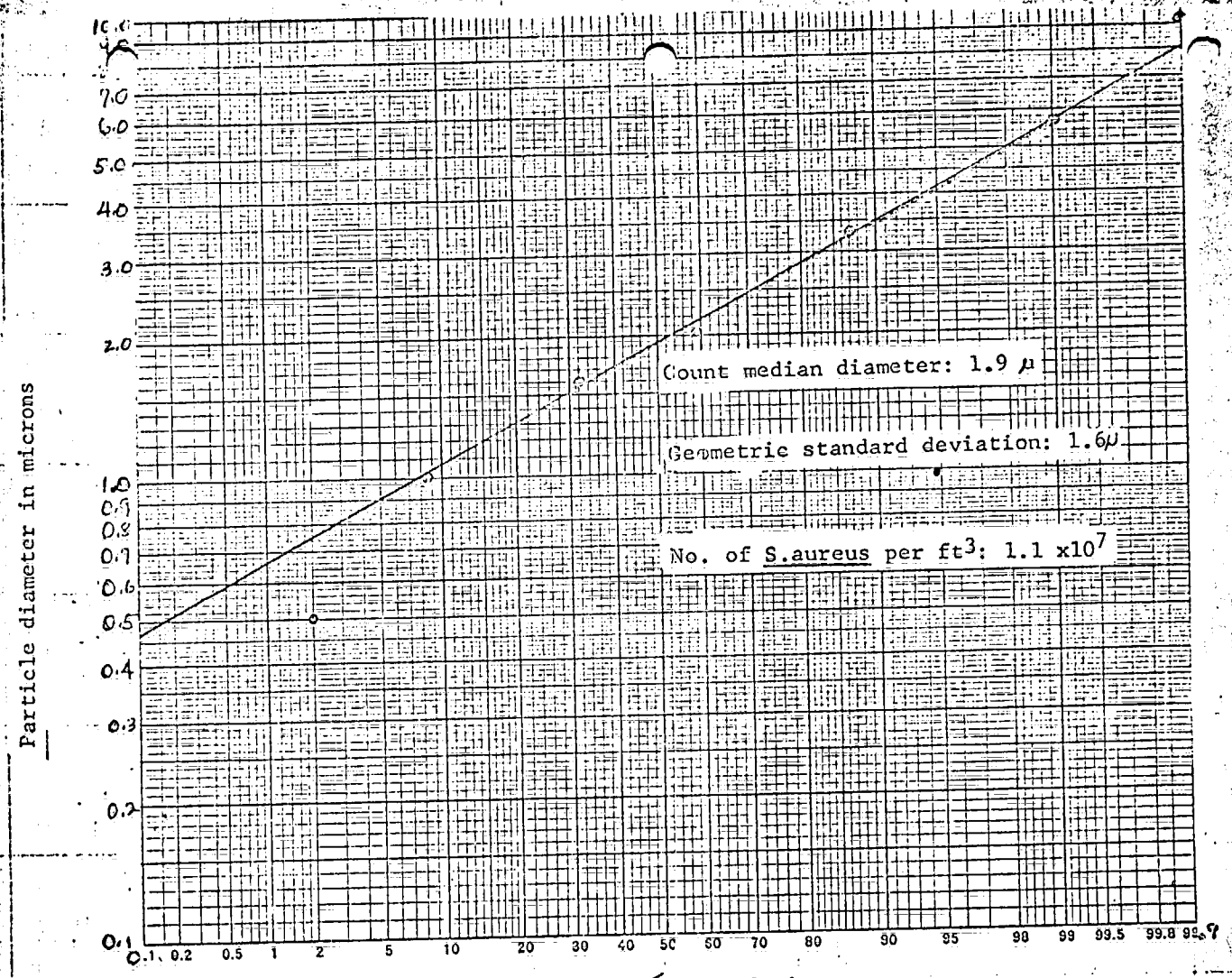

Fig.1 Particle size distribution of a staphylocccal aerosol.

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Table II Particle size distribution of the staphylococcal aerosols.

No. of S.aureus per ft^3 of air	Count Median Diameter μ	Geometric Standard Deviation μ	Cumulative % $\leq$ 3.3 μ
3.6×10^3	1.9	1.7	87
2.3×10^4	1.8	1.7	87
2.2×10^5	2.1	1.6	84
1.0×10^6	1.9	1.8	84
1.1×10^7	1.9	1.6	87

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Table III. The Numbers of Staphylococci Deposited in the Lungs of Mice of Various Weights Exposed to S. aureus at a Chamber Concentration of 2.1×10^4 organism/ft^3.

Mouse Weight (gms)	Number of S. aureus Deposited in the Lungs ($\times 10^3$)
24.2	70
25.1	76
33.5	93
34.5	93
35.0	110
37.2	120
39.1	125
42.0	139
44.8	145
45.0	139
48.0	150
50.0	168

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Table IV. Studies of Bacterial Clearance by Animals Housed for Various Periods of Time in The Laboratory Environment.

No. of Days Housed in Laboratory	No. of Bacteria Deposited in Lung	% Bacteria Cleared *
1	107,000	88
7	117,000	86
14	104,000	87
28	86,000	85

*Mean clearance values obtained from single studies.

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